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A Comparison of Effects of ABVD and ChlVPP Chemotherapeutic Protocols for Hodgkin's Disease on Rats' Epididiymal and Testicular Tissues

[1]A. Eyshi Oskooii, [2]L. Mohammad Gholizad, [2]S. Zare and [2]V. Nejati
[1]Department of Oncology, Emam Khomeyni Hospital, Urmia Medical Sciences University, Urmia, Iran
[2]Department of Biology, Science College, Urmia University, Urmia, Iran

Abstract: The goal of this study was to determine the effects of ABVD and ChlVPP chemotherapeutic protocols for Hodgkin's disease on the structure of testis and epididymis of male rat. After determining tolerance dose of drugs in pilot study, 24 male rats were divided to four groups: ABVD (doxorubicin, bleomycine, vinblastin, dacarbazine) group, ChlVPP (chlorambucil, vinblastin, procarbazine, prednisolone) group and two control groups one for each treatment group. One half of the lethal dose for 50% of population (LD_{50}) was used for treatment of animals in each protocol. Testes and epididymis tissues were examined for structural changes and serum testosterone level was measured by Lission (chemiluminescence method). Body weight, testis and epididymis weights, in treated rats were significantly less than their control groups specifically in ABVD group was less than ChlVPP group. Decreasing of mean diameter of seminiferous tubules, height of spermatogenic cells and diameter of epididymis in caput, corpus and cauda in ABVD group were significantly more than ChlVPP and control group. The serum testosterone level in ABVD group was significantly less than ChlVPP and control group. According to this study results, the ChlVPP had fewer impairment effects than ABVD on testis and epididymis tissue in tolerance doses on male rats' reproductive system. More clinical trial studies are suggested on Hodgkin's patients. With equal treatment effectiveness, it will be better to use the most reliable and safe treatment especially in young patients.

Key words: Lymphoma, gonadotoxicity, seminiferous, testosterone, rat

INTRODUCTION

Hodgkin's lymphoma (HL) is a rare malignancy, with an incidence of approximately 2.4 per 100 000 per annum in developed countries. Also, it comprises 6% of childhood cancers (Richard and Morgan, 2004). In these countries, the incidence of the disease is very low in young children, peaks in young adults, wanes in middle age then gradually increases again over the later decades (Kennedy *et al*., 1985; Armstrong *et al*., 1994).

Approximately 90 to 95% of children with Hodgkin lymphoma can be cured promoting increased attention to devising nonmorbid therapy of these patients (Donaldson and Link, 1991). A significant proportion of young patients have long term survival following chemotherapy. However, the major concern for patients is gonadal dysfunction with impaired reproductive capacity (Mushtaq *et al*., 2008). Germinal epithelium is particularly susceptible to injury by cytotoxic drugs and radiation due to its high mitotic rate (Jeff *et al*., 1988).

Although, there are some differences between human and animals' reproductive system, animal models are useful to study the damage rate of differentiation and morphogenesis of genital system (Pryor *et al*., 2000). Temporary or permanent infertility can be a common side effect of chemotherapy. In the testis, the cells of the germinal epithelium have the highest mitotic and meiotic indices and are thus most vulnerable to the toxic effects of chemotherapy (Schilsky *et al*., 1980; Roeser *et al*., 1978). Chemotherapy, through its effect on rapidly dividing cells, can cause aplasia (lack of development) of germinal epithelium which lines the seminiferous tubules reducing sperm production. This can often lead to low sperm count (oligospermia) or total absence of sperm in the semen (azoospermia) (Haskell, 2001). Male gonadal toxicity is a complex issue in Hodgkin lymphoma. Gonadal toxicity may manifest as infertility, lack of sexual development, small, atrophic testicles and sexual dysfunction. Infertility caused by azoospermia is the most common manifestation of gonadal toxicity (Viviani *et al*., 1991; Fitoussi *et al*., 2000). Common chemotherapeutic regimen in HD treatment are ABVD (Doxorubicin, Bleomycin, Vinblastin, Dacarbazine), ChlVPP (Chlorambucil, Vinblastine, Procarbazine, Prednisolone),

Corresponding Author: Lida Mohammad Gholizad, Department of Biology, Science College, Urmia University, Urmia, Iran
Tel: 00989143407009 Fax: 00984413660800

MOPP (Mustargent, Vincristin, Procarbazine, Prednisone) and also hybrid protocols (Weiner, 2000). Previous studies confirm that the MOPP has more gonadotoxic side effect than ABVD and ChlVPP. There has been no finding on the comparison of ABVD and ChlVPP effects on gonadal dysfunction. The goal of this study was to determine the effects of ABVD and ChlVPP chemotherapeutic protocols for Hodgkin's disease on the structure of testis and epididymis of male rat.

MATERIALS AND METHODS

Drugs: Drugs that were used in this study include: Doxorobicin (1 vial of 5 mL, Ebedoxo 10 mg 5 mL^{-1} each vial contains 10 mg of doxorubicin HCl, manufacturer: EBEWE Pharma Ges.b.H.Nfg.KG A-4866 Unterach, AUSTRIA, orderd by Sobhan chemotherapeutics Co. Tehran, Iran), Bleomycin sulfate (1 vial contains 15 mg (polency) of bleomycin sulfate, Nippon Kayakli Co. Ltd., 11-2.1-chome Fujimi, chiyoda ku. Tokyo, Japan), Vinblastin (1 vial + 1 solvent ampoule, each vial contains 10 mg of vinblastin sulfate and each solvent ampoule contains 5 mL 0.9% sodium chloride solution, Gedeon Richter Ltd., Budapest, Hungary in cooperation with Sobhan chemotherapeutics Co. Rasht, Iran), Dacarbazine (D.T.I.C. 100 mg main substance: Dacarbazin citrate, manufactured by medac GmbH, D-20354 Hamburg, ordered by I.P.I for the I.R. Iran), Chlorambucil (Leukeran tablet contains 2 mg Chlorambucil, manufactured by Heumann Pharma GmbH for Glaxo wellcome GmbH and Co. Bad Oldesloe, Germany), Procarbazine (Natulan 50 mg capsules, Sigma-TauS.p.A., farmaceutiche riunitevia pontina, Pomezia RM Italie) and Prednisolone (each scored tablet contains 5 mg of prednisolone, manufactured by Iran Hormone Co. Tehran, Iran).

Pilot study: Ten adult male rats were randomly divided into 2 groups of 5 rats each. One group was treated with ChlVPP protocol drugs and the second group was treated by ABVD protocol drugs with human equal doses. More than 50% of animals in each group died. We selected 10 male rats in two groups for the second time. Usage dose was decreased to half of human dose. ABVD treated group tolerated this dose but in ChlVPP treated group more than 50% of animals died. Another time, in one group, the 5 rats were treated by ChlVPP protocol drugs with 1/4 of human dose. Animals didn't die. Thus, usage dose was considered one half of the lethal dose for fifty percent of population (1/2 of LD_{50}) for ABVD and ChlVPP groups.

Animals and treatment: Twenty four adult male (215±20 g) and 48 virgin female (150±10 g) Wistar rats were purchased from Tehran Institute Pastour and housed under controlled light conditions (12:12 h light: dark) and 22°C±2 room temperature in the animal house of Urmia University. Animals were provided with food and water *ad libitum*. All animal studies were conducted in accordance with the principles and procedures outlined in the Guide to the Care and Use of Experimental Animals prepared by the Urmia Council on Animal Care. Males were randomly divided into 4 groups of 6 rats each. The rats from the control group (I) were gavaged on days 1 through 5 of week with 4.7 mL of normal saline 0.9% and ethanol 3% and on days 1 and 3 of week, the rats were given 0.05 mL of normal saline 0.9% by intra peritoneal injection. The treatment was performed in three cycles, every other week. The rats from the control group (II) were given 0.5 mL of normal saline 0.9% by intra peritoneal injection on day 2 of each week. The treatment was performed in 6 times, every week. The rats from the ChlVPP-treated group were gavaged on days 1 through 5 of week with 1/4 mg kg^{-1} of chlorambucil dissolved in ethanol 3% and 3.31/4 mg kg^{-1} of procarbazine dissolved in saline and 1.36/4 mg kg^{-1} of prednisolone dissolved in distilled water and on day 1 and 3 of week, the rats were given 1/4 mg kg^{-1} of vinblastin by intra peritoneal injection. The treatment was performed in three cycles, every other week. The rats from the ABVD-treated group were given 4.17/2 mg kg^{-1} of doxorubicin and 1.5/2 mg kg^{-1} of bleomycine dissolved in saline and 1/2 mg kg^{-1} of vinblastin and 62.5/2 mg kg^{-1} of dacarbazine dissolved in saline by intra peritoneal injection on day 2 of each week. The treatment was performed in six times, every week. This dose regimen was chosen based on the standard dose given to humans (Weiner, 2000), adjusted for surface area according to the following formula:

$$f\times \text{ mg kg}^{-1} = \text{mg m}^{-2}$$

where, f equals 6.0 for the rat (Bachmann *et al*., 1996). This dosing regimen differs from that used in humans in that humans ABVD protocol are treated in day 1 and 15 per cycle of 28 days. ChlVPP protocol are treated for the first 14 days per cycle of 28 days; due to physician advice and patient condition each course of treatment could vary from 4 to 6 cycles.

Tissue collection and preparation: At the end of the treatment, males were anesthetized and the ventral prostate, seminal vesicles, testis and epididymis were removed and weighed. Right testis and epididymis were rinsed with saline serum and fixed in a fixative solution. This fixative solution contains 100 mL Aldehyid formic and 9 g NaCl diluted in to 1 L of distilled water. The

tissues were dehydrated in EtOH alcohol series then embedded in paraffin. To evaluate spermatogenesis, the sections were stained with periodic acid-Schiff, according to the manufacturer's instructions (Sigma) (Bieber *et al*., 2006). Sections were viewed with a Carl Ziess LMD microscope (West Germany) containing CCD camera.

Serum testosterone level was measured automatically by Lission (chemiluminescence method).

Morphometrical analysis of tissues: Tissues were examined for structural changes. The following parameters were recorded: weight of testes and epididymis, diameter of somniferous tubules and epididymis in caput, corpus and cauda, height of spermatogenic and epithelial cells. The measurement of tissues was conducted by an equipped microscope with scaled objective.

Statistical analysis: Data were analyzed using the Independent t-test or Mann Witney U-test, according to their distribution. One-way ANOVA and Duncan test were used for comparing means of different groups. Data are presented as the mean plus or minus Standard Error of the Mean (SEM). The level of significance was considered $p<0.05$.

RESULTS

The normal distribution of all parameters has been tested by Kolmogrov-smirnov test. With One way ANOVA test, it was observed that means of rats' weight before treatment weren't significantly different between groups ($p = 0.47$, $F = 0.86$). By using Duncan test, we observed a significant difference in mean of weight between control and treatment groups after treatment ($F = 7.9$, $t = 0.001$). The mean of weight and serum testosterone level between ABVD and ChlVPP treatment group was compared by independent t-test. Results demonstrated considerable differences (Table 1). Comparison of different tissues weight between groups by One Way ANOVA test is shown in Table 2. Weight of testicles and epididymis were decreased in both treatment groups compared to their control groups that decreasing in ABVD treatment group was significantly more than ChlVPP treatment group.

While the serum testosterone level in ABVD group was considerably decreased compared to control group (Table 3), there were no significant difference between ChlVPP group and control group (Table 4). The serum testosterone level in ABVD group was significantly less than ChlVPP group (Table 5).

Diameter of somniferous tubules, height of spermatogenic cells and diameter of epididymis in caput, corpus and cauda in ABVD group were significantly less than control group.

Thickness of epididymis in caput, corpus, cauda and height of epithelial cells in corpus and cauda were significantly increased compared to control group (Table 3 and Fig. 1).

Height of spermatogenic cells was considerably decreased in ChlVPP group. Thickness of epididymis and

Table 1: Comparison of rats' weight in different groups

Study group	End point weight mean (g)	Mean of first weight (g)
ChlVPP control	256.6±5.1	196.6±5.6
ABVD control	266.6±11.1	212.5±17.1
ABVD	184.0±41	206.6±25.8
ChlVPP	226.0±18.4	206.0±13.6

Table 2: Comparison of tissue weight between different groups

Parameters	Mean of ABVD control	Mean of ABVD	Mean of ChlVPP control	Mean of ChlVPP	F	p-value
Weight of right testes and epididymis and ventral prostate and seminal vesicles (g)	6.07±0.3	1.38±0.7	5.7±0.3	3.50±1.3	48.7	0.000*
Weight of left testes and epididymis (g)	2.80±0.1	0.85±0.3	2.7±0.1	1.67±0.4	58.2	0.000*

*Statistically significant

Table 3: Comparison of testosterone level and different morphometrical parameters between ABVD and control group

Parameters	Mean of ABVD	Mean of ABVD control	t	df	p-value
Serum testosterone level (ng mL^{-1})	1.5±0.79	2.9±0.79	2.7	9.5	0.02*
Diameter of somniferous tubules (μm)	148.8±18.7	328.6±14.7	18.4	9.4	0.000*
Height of spermatogenic cells (μm)	41.1±8.6	108.9±8.1	14.05	9.9	0.000*
Diameter of epididymis in caput (μm)	120.6±30	338.5±13.2	16.2	6.8	0.000*
Diameter of epididymis in corpus (μm)	171.9±41.4	328.8±18.2	8.4	8.6	0.000*
Diameter of epididymis in cauda (μm)	224.7±23.8	491.8±15.7	22.8	8.6	0.000*
Thickness of epididymis in caput (μm)	39.3±6.2	25.5±3.8	4-.6	8.3	0.001*
Height of epithelial cells in caput (μm)	27.4±7.6	21.03±3.6	1-.8	7.1	0.1
Thickness of epididymis in corpus (μm)	54.0±10.8	28.7±2.7	-5.7	5.6	0.001*
Height of epithelial cells in corpus (μm)	35.8±3.9	22.7±3.1	-6.3	9.4	0.000*
Thickness of epididymis in cauda (μm)	61.2±23.8	21.6±2.8	-4.04	5.1	0.009*
Height of epithelial cells in cauda (μm)	32.1±5.5	11.5±0.8	9-	5.2	0.000*

*Statistically significant

Table 4: Comparison of testosterone level and different morphometrical parameters between ChlVPP and control group

Parameters	Mean of ABVD	Mean of ABVD control	t	df	p-value
Serum testosterone level (ng mL^{-1})	3.20±0.72	2.80±0.75	0.83	9.9	0.36
Diameter of somniferous tubules (μm)	295.70±62.4	332.40±11.3	-1.4	5.3	0.2
Height of spermatogenic cells (μm)	83.04±20.5	108.60±5.7	-2.9	5.7	0.02*
Diameter of epididymis in caput (μm)	283.70±84.1	339.10±18.1	-1.5	5.4	0.1
Diameter of epididymis in corpus (μm)	260.60±51.6	339.80±17.6	-3.5	6.1	0.01*
Diameter of epididymis in cauda (μm)	409.50±115.5	488.00±13	-1.6	5.1	0.1
Thickness of epididymis in caput (μm)	32.40±6.8	26.30±2.8	2.0	6.6	0.08
Height of epithelial cells in caput (μm)	25.20±4.1	21.60±2.6	1.7	8.5	0.1
Thickness of epididymis in corpus (μm)	40.70±14.9	29.80±2.2	1.7	5.2	0.13
Height of epithelial cells in corpus (μm)	29.80±7.1	23.70±2.5	1.9	6.2	0..09
Thickness of epididymis in cauda (μm)	47.40±16.6	21.07±2.6	3.8	5.2	0.01*
Height of epithelial cells in cauda (μm)	27.70±13.1	11.50±0.8	3.1	5.04	0.026*

*Statistically significant

Table 5: Comparison of testosterone level and different morphometrical parameters between ABVD and ChlVPP group

Parameters	Mean of ABVD	Mean of ABVD control	t	df	p-value
Serum testosterone level (ng mL^{-1})	1.5±0.97	3.2±0.72	-3.5	9.2	0.006*
Diameter of somniferous tubules (μm)	148.8±18.7	295.7±62.4	-5.5	5.8	0.002*
Height of spermatogenic cells (μm)	41.1±8.6	295.7±62.4	-1.6	6.7	0.003*
Diameter of epididymis in caput (μm)	120.6±30	283.7±84.1	-4.4	6.2	0.004*
Diameter of epididymis in corpus (μm)	171.9±41.4	260.6±51.6	-3.2	9.5	0.009*
Diameter of epididymis in cauda (μm)	224.7±23.8	409.5±115.5	-3.8	5.4	0.01*
Thickness of epididymis in caput (μm)	39.3±6.2	32.4±6.8	1.8	9.8	0.1
Height of epithelial cells in caput (μm)	27.4±7.6	25.2±4.1	0.64	7.6	0.53
Thickness of epididymis in corpus (μm)	54.06±10.3	40.7±14.9	1.7	8.9	0.1
Height of epithelial cells in corpus (μm)	35.8±3.9	29.8±7.1	1.7	7.7	0.1
Thickness of epididymis in cauda (μm)	61.2±23.8	47.4±16.6	1.1	8.9	0.27
Height of epithelial cells in cauda (μm)	32.1±5.5	27.7±13.1	0.74	6.7	0.48

*Statistically significant

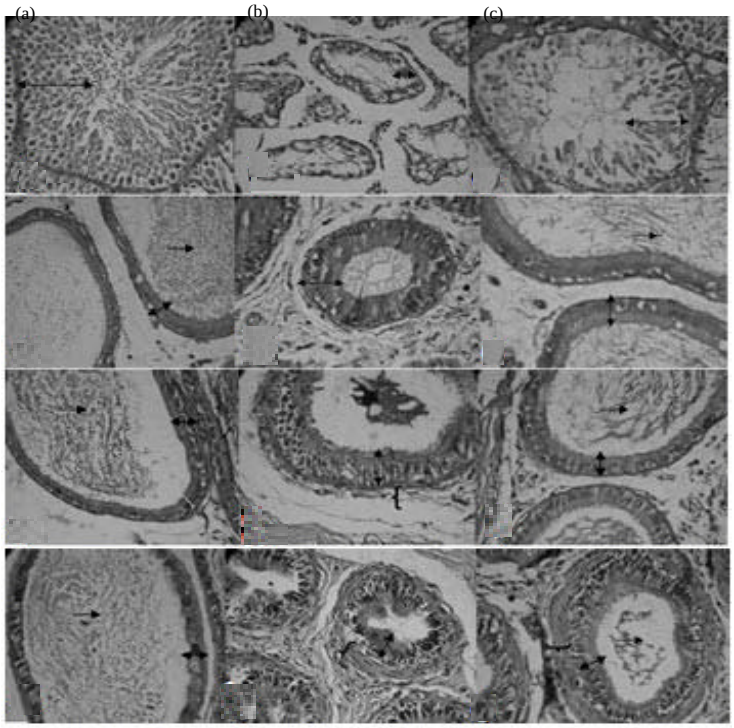

Fig. 1: The light microscope photographs of tissues staining with PAS, including to: (a) control, (b) ABVD and (c) ChlVPP groups. First row: cross section of testis shows a decrease in the number of spermatogenic cells in the somniferous tubules of treated groups. Section of epididymis in caput (Second row), corpus (Third row) and cauda (Forth row) shows differences in height of epithelial cells, thickness of epididymis and lumen

height of epithelial cells in cauda were significantly increased compared to control group (Table 4 and Fig. 1).

Comparison of ABVD and ChlVPP treatment groups showed that mean diameter of somniferous tubules, height of spermatogenic cells, diameter of epididymis in caput, corpus and cauda in ABVD group were significantly decreased compared to ChlVPP group. There was no significant difference between two groups in the other indices (Table 5 and Fig. 1).

DISCUSSION

According to authors Mackie *et al.* (1996), Papadakis *et al.* (1999), Whitehead *et al.* (1982), Howell and Shalet (1998), Shalet *et al.* (1981) Wallace *et al.* (1991), Watson *et al.* (1985), Heikens *et al.* (1996), Viviani *et al.* (1985) and Meistrich *et al.* (1982), Cytotoxic chemotherapy agents may produce permanent damage to the germinal epithelium of the testis. Cytotoxic treatment targets rapidly dividing cells and as a result, spermatogenesis can be disrupted after treatment (Meistrich *et al.*, 1982). Although, Leydig's and Sertoli's cell function is often preserved due to their very low proliferation index (Jeff *et al.*, 1988).

Twenty-seven of 30 men who received nitrogen mustard in the 1940s had significant testicular atrophy and absent spermatogenesis (Gilman, 1963). This study conform the Earlier study that testes atrophy was an important side effect after chemotherapy. This finding is in accordance with (Charak *et al.*, 1990), who have reported testicular atrophy was noticed in 89 (96.7%) patients (Gilman, 1963).

The germinal epithelium is far more sensitive to the effects of cytotoxic drugs than the Leydig cells. Impairment of leydig cells are usually limited to raised LH concentration with normal or low normal testosterone concentration (Howell *et al.*, 1999). While levels of serum testosterone following chemotherapy in prepubertal boys may be normal, testicular biopsies after combination therapy for acute lymphoblastic leukemia or Hodgkin's disease commonly show seminiferous tubular damage and interstitial fibrosis (Uderzo *et al.*, 1984). Shafford *et al.* (1993) has reported, 26 of 28 Hodgkin's patient who received chemotherapy in childhood, had elevated gonadotropin levels but normal serum testosterone levels and normal secondary sexual characteristics.

While we did not observe any significant changes in circulating testosterone levels, consistent with previous reports (Chapman, 1982). The production rate of testosterone may be significantly reduced in men with seminiferous tubular damage even if total testosterone levels are within the normal range (Booth *et al.*, 1987). Euan's study showed after chemotherapy although testosterone levels increased significantly from a baseline at 1 month, there were no changes at other time points over the 12 months (Euan *et al.*, 1997). Standard doses of chemotherapy do not lead to a significant deterioration of Leydig cell function in long term survivors. In contrast, high cumulative doses of chemotherapy cause a significant and persistent impairment of Leydig cell function (Gerl *et al.*, 2001). In this study, while the testosterone levels was significantly low in ABVD treated group, there wasn't significant change in ChlVPP group resulting in less impairment effects of this protocol.

Incidence of atrophic somniferous tubules following chemotherapy has reported by Goodpasture *et al.* (1988) and Schilsky *et al.* (1980). In this study, according to the study of Thomson *et al.* (2002) and Kreuser *et al.* (1987) decreasing in diameter of somniferous tubules resulting in disrupted spermatogenesis and damage to sensitive germinal epithelium was showed in both treated group. In despite of decreasing diameter of epididymis in caput, corpus and cauda the thickness of epididymis was significantly increased in ABVD treated group compared to ChlVPP group. Increasing height of epithelium, connective tissue and smooth muscle lead to increasing the thickness of epididymis and respectively decreasing the lumen space of epididymis. These changes in ChlVPP treated group were less than ABVD group.

There was no comparative morphometrical and histological study between these protocols and due to high mortality in rats, we were obliged to decrease the dosage of ChlVPP protocol to 1/4 of human dose. It might affect on present results, however, decreasing drug dose under LD_{50} is common in experimental studies.

CONCLUSION

ChlVPP protocol has fewer side effects than ABVD on reproductive tissues and testosterone level. We suggest selecting ChlVPP protocol as a choice treatment for Hodgkin's disease after evaluation of this protocol on clinical trial studies.

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